PATENT APPLICATION
Atty. Docket No. 702-001525

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Group Art Unit Not Yet Assigned	:	
In re application of	:	
	:	**NEW STAPHYLOKINASE**
Desiré José COLLEN	:	**DERIVATIVES**
Serial No. Not Yet Assigned	:	
Examiner – Not Yet Assigned	:	

Pittsburgh, Pennsylvania
November 29, 2000

PRELIMINARY AMENDMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Prior to examination on the merits, please amend the above-identified application as follows:

IN THE SPECIFICATION:

On page 3, after the title and before line 1, insert the following headings and paragraph:

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional of United States Patent Application Serial No. 09/020,018, filed February 6, 1998, which is a continuation-in-part of United States Patent Application Serial No. 08/784,971, filed January 16, 1997, now United States Patent No. 5,951,980, issued September 14, 1999, which is a continuation-in-part of United States Patent Application Serial No. 08/499,092, filed July 6, 1995, which is a continuation-in-part of United States Patent Application Serial No. 08/371,505, filed January 11, 1995, now United States Patent No. 5,695,754, issued December 9, 1997.

BACKGROUND OF THE INVENTION

1. Field of the Invention

On page 3, before line 6, insert the following heading:

2. Description of the Related Art

On page 4, before line 18, insert the following heading:

SUMMARY OF THE INVENTION

On page 5, before line 20, insert the following headings and paragraphs:

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a protein sequence of wild-type staphylokinase, SakSTAR (SEQ ID NO: 10). Numbering starts with the NH_2-terminal amino acid of mature full length staphylokinase.

Fig. 2 is a time course of neutralizing activities (left panel) and specific IgG against administered agent (right panel) following intra-arterial infusion of SakSTAR (open circles, n=9), SakSTAR (K74A) (closed circles, n=11) or SakSTAR (K74A,E75A,R77A) (open squares, n-6) in patients with peripheral arterial occlusion. The data represent median values and interquartile ranges, in μg/ml.

Fig. 3 is a protein sequence of wild-type staphylokinase, SakSTAR with indicated amino acid substitutions.

Squares: single amino acid substitutions; circles: combined (2 to 3) amino acid to Ala substitutions.

Fig. 4 shows temperature stability of SakSTAR, (A); SakSTAR (K74Q, E80A, D82A, K130T, K135R), (B); SakSTAR (E65D, K74R, E80A, D82A, K130T, K135R), (C); and SakSTAR (K35A, E65D, K74Q, E80A, D82A, K130T, K135R), (D).

(○): 4°C; (●): 20°C; (∇): 37°C; (▼): 56°C; (□): 70°C.

Fig. 5 is a time course of neutralizing activities (left panel) and specific IgG against administered agent (right panel) following intra-arterial infusion of SakSTAR (circles, n=15), SakStar (K74Q, E80A, D82A, K130T, K135R) (squares, n=6) or SakSTAR (E65D, K74R, E80A, D82A, K130T, K135R) (triangles, n=6) in patients with peripheral arterial occlusion. The data represent median values and 15-85 percentile ranges, in μg/mL.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

On page 9, please delete the first complete paragraph and insert the following replacement paragraph:

The plasmids encloding SakSTAR(K35A,E38A,K74A,E75A), SakSTAR(E38A,E75A,R77A), SakSTAR(E38A,E75A), SakSTAR(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E80A), SakSTAR(D82A), SakSTAR(E75A,D82A), SakSTAR(K74A) and SakSTAR(E75A) were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24), using Vent DNA polymerase (New England Biolabs, Leusden, The Netherlands), and available or generated *sakSTAR* variants as template. Two fragments were amplified by PCR, the first one starting from the 5' end of the *staphylokinase* gene with primer 5'-CAGGAAACAGAATTCAGGAG-3' (SEQ ID NO: 1) to the region to be mutagenized (forward primer), the second one from the same region (backward primer) to the 3' end of the *staphylokinase* gene with primer 5'-CAAAACAGCCAAGCTTCATTCATTCAGC-3' (SEQ ID NO: 2).

On page 19, please delete the first complete paragraph and insert the following replacement paragraph:

The variants SakSTAR(Y17A,F18A), SakSTAR(F104A), SakSTAR(F111A), SakSTAR(Y9A), SakSTAR(Y91A), SakSTAR(Y92A), SakSTAR(I87A), SakSTAR(I106A) and SakSTAR(I120A) were constructed with the Chameleon Double-Stranded Site-Directed Mutagenesis kit from Stratagene (La Jolla, USA), using the *pMEX.SakSTAR* vector as tmplate, and following instructions of the supplier. The mutagenic oligonucleotides (not shown) were used in combination with the selection-primer LY34 5' CAAAACAGCCGAGCTTCATTCATTCAGC (SEQ ID NO: 3), which destroys the unique *Hind*III site located 3' to the staphylokinase encoding gene in *pMEX.SakSTAR* and allows to counter-select the non-mutant progeny by HindIII digestion. The deletion of the *Hind*III site was in most cases correlated with the presence of the desired mutation introduced by the mutagenic oligonucleotide. The variant SakSTAR(I133A), was constructed by performing a polymerase chain reaction on the *pMEX.SakSTAR* plasmid using the primer 818A located at the 5' end of the *sak*STAR gene (5' CAGGAAACAGAATTCAGGAG) (SEQ ID NO: 1) and the mutagenic primer LY58 (5' TTCAGCATGCTGCAGTTATTTCTTTTCTGCAACAACC TTGG) (SEQ ID NO: 4). The amplified product (30 cycles: 30 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C) was purified, digested with EcoRI and PstI, and ligated into the corresponding sites of *pMEXSakSTAR*.

On page 20, please delete the first complete paragraph and insert the following replacement paragraph:

The plasmids encoding all the other variants listed in Table 3 were contructed by direct PCR or by the spliced overlap extension polymerase chain reaction (SOE-PCR)(24) using *pMEX.SakSTAR* or available plasmids encoding SakSTAR variants as template. Two

fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the *staphylokinase* gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled to gether in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindII and ligated into the corresponding site of *pMEX.SakSTAR*. For each construction, the sequence of the variant was confirmed by sequencing the entire *Sak*STAR coding region.

On page 32, please delete the first complete paragraph and insert the following replacement paragraph:

The variants SakSTAR(K102C) and SakSTAR(K109C), were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using *pMEX.SakSTAR* encoding SakSTAR as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the *staphylokinase* gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp (for the contruction of K102C: TAT GAT AAG AAT TGC AAA AAA GAA GAA (backward) (SEQ ID NO: 6) and TTC TTC TTT TTT GCA ATT CTT ATC ATA (forward) (SEQ ID NO: 7) for the construction of K109C: AAA AAG AAG AAA CGT GCT CTT TCC CTA (backward) (SEQ ID NO: 8) and TAG GGA

AAG AGC ACG TTT CTT CTT TTT (forward) (SEQ ID NO: 9). The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of *pMEX.SakSTAR*. For each construction, the sequence of the variant was confirmed by sequencing the entire *Sak*STAR coding region.

IN THE CLAIMS:

Please cancel claims 1-12 and add the following new claims 13-21:

13. (New) A staphylokinase derivative having essentially the amino acid sequence as depicted in Figure 3 in which one or more encircled or boxed amino acids have been replaced by another amino acid thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase and further incorporating one or more polyethylene glycol groups.

14. (New) The staphylokinase derivative of claim 13 in which one polyethylene glycol group is coupled in position 102 thereon.

15. (New) The staphylokinase derivative of claim 13 in which the staphylokinase specific activity of the derivative is at least 50 percent that of wild type staphylokinase.

16. (New) Staphylokinase derivative SakSTAR(K35X,G36X,E65X,K74X, E80X,D82X,K102X,E108X,K109X,K121X,K130X,K135X,K136X,+137X) having the amino acid sequence as depicted in Figure 1 in which the amino acids Lys in position 35, Gly

in position 36, Glu in position 65, Lys in position 74, Glu in position 80, Asp in position 82, Lys in position 102, Glu in position 108, Lys in position 109, Lys in position 121, Lys in position 130, Lys in position 135 and/or Lys in position 136 have been replaced with other amino acids and/or in which one amino acid has been added at the COOH-terminus, thus altering the immunogenicity after administration in patients, and further incorporating at least one polyethylene glycol group.

17. (New) Staphylokinase derivatives listed in Tables 1, 2, 3, 4, 5, 6, 7 and 8, having the amino acid sequence depicted in figure 3 in which the boxed or encircled amino acids have been replaced by other amino acids thus reducing the absorption of SakSTAR specific antibodies from plasma of patients treated with staphylokinase, without reducing the specific activity, the derivative further incorporating at least one polyethylene glycol group.

18. (New) Method for producing the staphylokinase derivatives as claimed in claim 13, comprising the steps of:

a. preparing a DNA fragment comprising at least the part of the coding sequence of staphylokinase that provides for its biological activity;
b. performing in vitro site-directed mutagenesis on the DNA fragment to replace one or more codons for wild-type amino acids by a codon for another amino acid;
c. cloning the mutated DNA fragment in a suitable vector;
d. transforming or transfecting a suitable host cell with the vector; and
e. culturing the host cell under conditions suitable for expressing the DNA fragment.

19. (New) Method as claimed in claim 18, wherein the DNA fragment is a 453 bp *Eco*RI-*Hin*dIII fragment of the plasmid *pMEX602sakB*, the *in vitro* site-directed mutagenesis is performed and the mutated DNA fragment is expressed in *E. coli*.

20. (New) Pharmaceutical composition comprising at least one of the staphylokinase derivatives as claimed in claim 13 together with a suitable excipient.

21. (New) Pharmaceutical composition as claimed in claim 20 for treating arterial thrombosis.

REMARKS

The specification has been amended to place the application in conformance with standard United States Patent practice.

Claims 1-12 have been cancelled. New claims 13-21 have been added further to define the invention, over U.S. Application Serial No. 09/020,018 of which the application filed herewith is a divisional application.

Examination and allowance of new claims 13-21 are respectfully requested.

Respectfully submitted,

WEBB ZIESENHEIM LOGSDON
ORKIN & HANSON, P.C.

By _____
Barbara E. Johnson
Registration No. 31,198
Attorney for Applicant
700 Koppers Building
436 Seventh Avenue
Pittsburgh, PA 15219-1818
Telephone: 412/471-8815
Facsimile: 412/471-4094

MARKED UP AMENDED SPECIFICATION PARAGRAPHS

Page 9, first complete paragraph

The plasmids encloding SakSTAR(K35A,E38A,K74A,E75A), SakSTAR(E38A,E75A,R77A), SakSTAR(E38A,E75A), SakSTAR(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E80A), SakSTAR(D82A), SakSTAR(E75A,D82A), SakSTAR(K74A) and SakSTAR(E75A) were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24), using Vent DNA polymerase (New England Biolabs, Leusden, The Netherlands), and available or generated *sakSTAR* variants as template. Two fragments were amplified by PCR, the first one starting from the 5' end of the *staphylokinase* gene with primer 5'-CAGGAAACAGAATTCAGGAG-3' (SEQ ID NO: 1) to the region to be mutagenized (forward primer), the second one from the same region (backward primer) to the 3' end of the *staphylokinase* gene with primer 5'-CAAAACAGCCAAGCTTCATTCATTCAGC-3' (SEQ ID NO: 2).

Page 19, first complete paragraph

The variants SakSTAR(Y17A,F18A), SakSTAR(F104A), SakSTAR(F111A), SakSTAR(Y9A), SakSTAR(Y91A), SakSTAR(Y92A), SakSTAR(I87A), SakSTAR(I106A) and SakSTAR(I120A) were constructed with the Chameleon Double-Stranded Site-Directed Mutagenesis kit from Stratagene (La Jolla, USA), using the *pMEX.SakSTAR* vector as tmplate, and following instructions of the supplier. The mutagenic oligonucleotides (not shown) were used in combination with the selection-primer LY34 5' CAAAACAGCCGAGCTTCATTCATTCAGC (SEQ ID NO: 3), which destroys the unique *Hind*III site located 3' to the staphylokinase encoding gene in *pMEX.SakSTAR* and allows to counter-select the non-mutant progeny by HindIII digestion. The deletion of the *Hind*III site was in most cases correlated with the presence of the desired mutation introduced by the

mutagenic oligonucleotide. The variant SakSTAR(I133A), was constructed by performing a polymerase chain reaction on the *pMEX.SakSTAR* plasmid using the primer 818A located at the 5' end of the *sak*STAR gene (5' CAGGAAACAGAATTCAGGAG) (SEQ ID NO: 1) and the mutagenic primer LY58 (5' TTCAGCATGCTGCAGTTATTTCTTTTCTGCAACAACCTTGG) (SEQ ID NO: 4). The amplified product (30 cycles: 30 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C) was purified, digested with EcoRI and PstI, and ligated into the corresponding sites of *pMEXSakSTAR*.

Page 20, first complete paragraph

The plasmids encoding all the other variants listed in Table 3 were contructed by direct PCR or by the spliced overlap extension polymerase chain reaction (SOE-PCR)(24) using *pMEX.SakSTAR* or available plasmids encoding SakSTAR variants as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the *staphylokinase* gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled to gether in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindII and ligated into the corresponding site of *pMEX.SakSTAR*. For each construction, the sequence of the variant was confirmed by sequencing the entire *Sak*STAR coding region.

Page 32, first complete paragraph

The variants SakSTAR(K102C) and SakSTAR(K109C), were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using *pMEX.SakSTAR* encoding SakSTAR as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the *staphylokinase* gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp (for the contruction of K102C: TAT GAT AAG AAT TGC AAA AAA GAA GAA (backward) (SEQ ID NO: 6) and TTC TTC TTT TTT GCA ATT CTT ATC ATA (forward) (SEQ ID NO: 7) for the construction of K109C: AAA AAG AAG AAA CGT GCT CTT TCC CTA (backward) (SEQ ID NO: 8) and TAG GGA AAG AGC ACG TTT CTT CTT TTT [(forward)] (forward) (SEQ ID NO: 9). The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of *pMEX.SakSTAR*. For each construction, the sequence of the variant was confirmed by sequencing the entire *Sak*STAR coding region.